J. Cell Biol

MUCOPOLYSACCHARIDES IN THE PULMONARY SURFACTANT HYPOPHASE. AN ELECTRON MICROSPICAL STUDY OF RAT LUNGS TREATED WITH RUTHENIUM RED. *

Branislav Vidic, Margit Hamosh and Paul Hamosh
From the Departments of Anatomy and Physiology
and Biophysics , Georgetown University School
of Medicine, Washington, D. C. 20007

Abstract

The alveoli of the lung are coated by surfactant, composed of phospholipids, proteins and mucopolysacchrides. Morphologically, it consists of an epiphase and a hypophase. The location of mucopolysacchrides in the surfactant has been identified through the use of Ruthenium Red (RR), a stain specific for mucoid substances. Electron microscopic analysis showed RR-positive material in association with the external surface of "tubular myelin", a component of surfactant hypophase. These observations support the hypothesis that the surfactant system consists of sliding layers of phospholipid, separated by mucopolysacchrides and protein.

Introduction

The surfactant system, an osmiophilic superficial layer covering the alveolar surfaces, is usually subdivided into two layers. There is a thin superficial layer called the epiphase, and a layer of varying thickness, found between the cell membrane and the epiphase, called the "base layer" (4) or hypophase (12). There is general agreement that the epiphase consists of dipalmitoyl

lecithin (DPL) and other components of the lung surfactant system. The hypophase, however, is morphologically complex, and its chemical composition is not well understood. The hypophase is irregular in thickness. In well preserved specimens, it seems to fill the space between the smooth epiphase layer and the irregular cell surface. It is amorphous in appearance, with a varying amount of vesicular profiles, and in some places it forms intricate patterns called "tubular myelin".

Histochemical and biochemical studies of lung washings show, in addition to phospholipids and mucopolysacchrides, significant amounts of protein and glucoprotein. Since these substances do not form mono- or bi- layers, as does DPL, they probably originate from the hypophase. The presence of mucopolysacchrides in the hypophase is probably essential for the proper function of the surfactant system. In the present study, Ruthenium Red (RR) was used in an effort to illustrate the spatial relationship of mucopolysacchrides to other components of the surfactant system.

Materials and Methods

Adult, healthy Sprague-Dawley rats of both sexes were injected intraperitoneally with lethal doses of Pentobarbital. Following thoracolaparotomy and removal of the diaphragm along the costal attachments, pieces of lung were excised from both

lower lobes. Some samples were double-fixed by immersion, whereas others were first immersed for two hours at 4°C in a solution (8) made of equal quantities of glutaraldehyde-formaldehyde stock mixture (6), 0.1M sodium cacodylate buffer pH 7.4, and RR stock solution (1,500 ppm in water). A brief rinse of the tissue in cold buffer was followed by a second fixation and an additional application of RR (equal quantities of a 5% aqueous solution of osmium tetroxide, 0.1M sodium cacodylate buffer pH 7.4, and RR stock solution) at 4°C for one hour. In some animals the trachea was transected and connected to a small rodent respirator by a cannula. A vascular cannula was introduced through the right ventricle and connected to a peristaltic pump. Unimpeded venous outflow was secured by wide excision of the left auricle. The specimen was suspended from a force transducer in a thermal chamber at 37°C. Both lungs were ventilated in the chamber one to three times per minute at one ml tidal volume. They were then perfused for one to two hours with the solution of aldehydes and RR, prior to the removal of samples from both lower lobes. In some instances, the perfusion was continued using first the buffer solution and then the second fixative. All specimens were stained en block at room temperature for one hour in 1% uranyl acetate in maleate buffer pH 6.0, dehydrated and embedded in Epon (7). Thick sections (0.5 to 1.0μ) were used to evaluate the penetration of RR into the blocks. Thin sections were treated with heavy metal salts (14,15) and examined in an AEI-EM 801 electron microscope.

Results and Discussion

RR does not readily penetrate the cellular interior. To the small extent that it does penetrate, it is bound to the lipid droplets or the internal aspects of the endoplasmic reticulum membranes. Some investigators (5) attribute this location to cross-bindings from the hexavalent RR to the negatively charged part of the juxtaposed phospholipid layers comprising endoplasmic reticulum. When stained by RR, the cysternal configuration appears as a pentalamellar form in thin sections (5). However, most RR-positive material is found outside the cell, in the cellular coat, where it is attracted by polyanionic complexes of acidic syalo- and/or sulfo- mucinous molecules. These molecular complexes are probably made up of polypeptides and polysacchrides which are bound in varying proportions. Biological membranes do not provide an adequate number of anionic arms for binding a hexavalent cation such as RR (8). Thus, when RR is used alone, phospholipid mono- or bi- layers are not stained.

The difficultues encountered in labeling the inside of the alveoli by intra-vascular administration of RR, were due to a lack of penetration by the dye across the pulmonary endothelium. The surfactant system (4) was almost entirely destroyed during fixation by immersion. For these reasons, an attempt was made to first use perfusion fixation with a solution of aldehyde-RR, and then to immerse the speciman into RR-containing fixative. Although it was impossible to preserve the entire surfactant

1003545851

layer within any single alveolus, there were sufficient locations where the epiphase and hypophase were satisfactorily preserved.

Figure 2 shows the surfactant epiphase, a monolayer coating a type II alveolar cell. An indentation in the adjacent cell is filled with an amorphous material, which has a vesicular appearance toward the monolayer. A similar area of hypophase is shown in Figure 3. The proximity of the vesicles to the monolayer is remarkable. It immediately raises the unresolved question: Is the hypophase a precursor of the monolayer or a product of its degradation?

Figure 4 shows some "tubular myelin" coiled up in the hypophase. This configuration might either represent a just extruded lamellar body or some worn out surfactant debris. Phospholipids do form these configurations in aqueous solution regardless of their functional viability. Figure 5 shows all forms of hypophase, and might support the evidence that the hypophase is synthesized de novo, because of the presence of active transporting vesicles. Endo- and pinocytosis by type II cells occurs rarely, if at all. Sometimes the entire hypophase consists of "tubular myelin" (Fig. 6). These networks are composed of typical trilaminar membranes with a diameter of about 45 Å that intersect at approximately right angles. The resulting gridholes range in diameter from 313 Å to 571 Å. This range is compatible with the values obtained for lattice-like membranes of the newborn rabbit after pharyngeal deposition of surfactant (11).

Assuming that three distinctly identifiable components of

the hypophase (amorphous, vesicular, "tubular myelin") are of similar biochemical composition, their appearance indicates a complex physico-chemical environment and not a simple aqueous phase (11).

Surfactant treated with fixatives containing RR. The perfusion-fixation technique precipitated RR only on the cellular coats of endothelial cells, as far centrifugally as the tight junctions. The immersion-fixation method facilitated binding of RR to cellular coats of epithelial cells as far as their tight junctions. In both instances, the cellular coats bind RR molecules (Figs. 1,7) along an approximately 320 Å wide zone, for comparison see (1). Contrary to previous findings (10), no intracellular binding sites for RR were found in this study, with the exception of lipid globules inside the macrophage, and cytoplasmic inclusions of the mast cell.

Two distinct parts of the pulmonary surfactant are repeatedly observed to bind a great quantity of RR molecules: 1) the zone immediatly adjacent to the monolayer (Fig. 8); and 2) the lattice-like membranous networks of the surfactant hypophase (Figs. 9, 10). In the former location, RR reveals a "fuzzy" layer with a diameter similar to that of the cellular coat of either the endothelial or the epithelial cells. Some of the vesicles in the hypophase are also stained with RR, but only externally.

In the lattice-arranged membranes, the hexavalent cation binds heavily along the boundaries of the gridholes. In this

instance, the labelled complexes seem to be associated with the external faces of the crossing membranes, while the membranous interior remains label-free. Consequently, the membrane itself, in this preparation, appears as a negatively stained image with RR-positive material @ 90 to 150 Å in diameter, paralleling it on both sides. The opacity of this material is particularly pronounced at the crossing points of perpendicularly intercepting membranes. These findings support some speculations on the composition and organization of the surfactant system (2). The surfactant epiphase is a monolayer of phospholipid and has its chain (hydrophobic) poles oriented toward the alveolar surface and its hydrophilic poles toward the cell surface. Therefore, since RR reacts only with the hydrophilic phase, it stains only the inside of the monolayer (Fig. 8). The external cell coats are well-stained only where not protected by surfactant. Even more significant is the presence of RR-positive material, presumable mucopolysacchrides, at the cross-over points of the lattice work. Assuming that the surfactant system consists of several layers of surface active material which glide on each other during respiration (13), it is difficult to visualize such motion without some other material interposed between the adjacent polar surfaces. It was suggested that the surfactant system consists of layers of saturated DPL, stabalized by Ca++ and Na+ ions (9), polysacchrides, sialo and/or other mucins (3), and other phospholipids. The location of

1003545854

RR-positive material between layers of phospholipids or separating these layers from the cell surface lends considerable credence to the above speculations.

FOOTNOTE

* This investigation was supported by Grants from the
Washington Heart Association and The Council for Tobacco
Research, U.S.A.

1003545856

REFERENCES

1. Adamson, I.Y.R., and D.H. Bowden. 1970. The surface complexes of the lung. A cytochemical partition of phospholipid surfactant and mucopolysacchrides. Am. J. Pathol. <u>61</u>: 359.-368

✓2. Clements, J.A. 1973. Composition and properties of pulmonary surfactant. <u>In Respiratory Distress Syndrome</u>. C.A. Villee, D.B. Villee, and J. Zuckerman, editors. Academic Press, New York. 77.

3. Fishman, A.P., and G.G. Pietra. 1974. Handling of bioactive materials by the lung. New England J. Med. <u>291</u>: 884.-890

4. Gil, J., and E.R. Weibel. 1969/70. Improvements in demonstration of lining layer of lung alveoli by electron microscopy. Resp. Physiol. <u>8</u>: 13.-36

5. Howell, J.N. 1974. Intracellular binding of ruthenium red in frog skeletal muscle. J. Cell Biol. <u>62</u>: 242.-247

6. Ito, S., and M.J. Karnovsky. 1968. Formaldehyde-gluteraldehyde fixatives containing trinitro compounds. J. Cell Biol. <u>39</u>: 168A.-169A

7. Luft, J.H. 1961. Improvements in epoxi resin embedding methods. J. Biophys. Biochem. Cytol. <u>9</u>: 409.-414

8. Luft, J.H. 1971. Ruthenium red and violet I chemistry, purification methods of use for electron microscopy and mechanism of action. Anat. Rec. <u>171</u>: 347.-368

9. Morgan, T.E. 1971. Pulmonary surfactant. New England J. Med. <u>284</u>: 1185-1193

10. O'Hare, K.H. 1974. Fine structural observations of ruthenium red binding in developing and adult rat lung. Anat. Rec. <u>178</u>: 267.-288

11. Robertson, B. and G. Enhorning. 1974. The alveolar lining of the premature newborn rabbit after pharyngeal deposition of surfactant. Lab. Invest. <u>31</u>: 54.-59

✓12. Scarpelli, E.M. 1968. The Surfactant System of the Lung. Lea and Febiger, Phildelphia.

13. Tierney, D.G. 1974. Lung metabolism and biochemistry. Ann. Rev. Physiol. <u>36</u>: 209.-231

14. Venable, J.H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. <u>25</u>: 407.-408

REFERENCES (Continued)

15. Watson, M.L. 1958. Staing of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4: 475-478

PLATE I

EXPLANATION OF FIGURES

1. In this view the depth of penetration by RR through the capillary endothelium and intercellular spaces (S) is demonstrated. The binding of RR to the glucoproteins of the cellular coat is apparent. Also shown is the labelling of some pinocytotic figures (arrowhead). The basal lamina supporting the endothelial cells is indicated by an arrow. Double fixation with RR by way of pulmonary artery, uranyl acetate and lead citrate stain. X 18,900

2. A close relationship between the surfactant monolayer (arrow) and the villar projections of type II pneumocyte (P) is demonstrated in this section. In an indented part of the alveolus, the amorphous (A) and vesicular (arrowhead) layers of the hypophase are also shown. Uranyl acetate and lead citrate stain. X 35,000

3. Another indentation of the alveolus between the endothelial cells (E) is primarily occupied by the amorphous phase (A) of the surfactant. This material is covered toward the alveolar lumen (L) by a discrete layer of vesicle-like profiles (arrow) and the surfactant monolayer (arrowhead). Uranyl acetate and lead citrate stain. X 67,000

4. The surfactant hypophase shows, in addition to the amorphous material (A) and the vesicular layer (arrows), a large myelin-like figure. Note the distribution of vesicles in relation to the figure. The surfactant monolayer is indicated by an arrowhead. Uranyl acetate and lead citrate stain. X 24,000

5. From the endothelial cells (E) toward the alveolar lumen (L), the surfactant hypophase consists of the following layers: amorphous (A); vesicular (V); and lattice-arranged membranes (M). In similar sections the vesicular layer appears as a transitional material between the amorphous and membranous phases. Note also the structure of the

PLATE I (Continued)

material contained in several endothelial t nsporting vesicles
(arrows) in comparision to the amorphous phase. Uranyl acetate
and lead citrate stain. X 45,000

PLATE II

EXPLANATION OF FIGURES

6.In some instances, as is demonstrated in this section, the entire depth of the surfactant hypophase is represented by the lattice-arranged membranes measuring @ 2.4μ in thickness. The surfactant monolayer is indicated by an arrowhead, and the basal lamina supporting the endothelial cells (E) by an arrow. Uranyl acetate and lead citrate stain. X 17,000

7.Once the surfactant monolayer becomes disrupted by the fixation procedure, RR binds extensively to the cellular coats, covering the surface of both types of pneumocytes. No intracellular organelle of type II pneumocyte shows the reaction product. Double fixation with RR by immersion. X 8,000

8.Because of the immersion fixation procedure, the surfactant monolayer (arrowhead), although still properly oriented, is dis-associated in this section from the surface of type II pneumocytes (arrow). The binding sites of RR correspond to the hydrophilic poles of the monolayer and the cellular coat, respectively. Double fixation with RR by immersion, uranyl acetate and lead citrate stain. X 23,000

9.Conventional electron microscopy reveals certain material, similar to that described for the cellular coat, in association with the lattice-arranged membranes of the hypophase. This material is especially concentrated at the crossing points between membranes (arrow). Uranyl acetate and lead citrate stain. X 126,000

PLATE II (Continued)

10. RR binds strongly to the opaque material associated with the lattice-arranged membranes of the hypophase. In this instance, the crossing membranous bilayers appear as negative images. Double fixation with RR by immersion, uranyl acetate and lead citrate stain. X 150,000

FOOTNOTE